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FILING DATE: May 18, 1998



By Authority of the
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For and on behalf of RWS Translations Ltd.

The 30th day of September 1998



ST 98009

France

PATENT OF INVENTION

NEW AGENTS FOR TRANSFERRING NUCLEIC ACIDS, COMPOSITIONS
CONTAINING THEM AND THEIR APPLICATIONS,
THEIR PREPARATION AND THEIR USE FOR THE
TRANSFER OF NUCLEIC ACIDS INTO CELLS

RHONE-POULENC RORER S.A.

ABSTRACT

The present invention relates to new agents for transferring nucleic acids into cells. These new agents for transferring nucleic acids are more particularly related to the lipopolyamine family, and comprise at least one cyclic amidine functional group. These agents are useful for the transfection of nucleic acids of interest into different cell types either *in vitro*, *in vivo* or *ex vivo*.

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COMPOUNDS, THEIR PREPARATION AND THEIR USE FOR THE
TRANSFER OF NUCLEIC ACIDS INTO CELLS

The present invention relates to new agents for transferring nucleic acids into cells. These new agents for transferring nucleic acids are more particularly related to the lipopolyamine family, and comprise at least one cyclic amidine functional group. These agents are useful for the transfection of nucleic acids into different cell types either *in vitro*, *ex vivo* or *in vivo*.

With the development of biotechnology, the possibility of effectively transferring nucleic acids into cells has become a necessity. It may involve the transfer of nucleic acids into cells *in vitro*, for example, for the production of recombinant proteins, or in the laboratory for studying the regulation of the expression of genes, the cloning of genes, or any other manipulation involving DNA. It may also involve the transfer of nucleic acids into cells *in vivo*, for example for the creation of transgenic animals, the production of vaccines, labelling studies or also therapeutic approaches. It may also be the transfer of nucleic acids into cells *ex vivo*, in approaches including bone marrow transplants, immunotherapy or other methods involving the transfer of genes into cells collected from an organism for the purpose of their subsequent readministration.

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Various types of synthetic vector have been developed to improve the transfer of nucleic acids into cells. Among these vectors, cationic lipids possess advantageous properties. These vectors consist of a 5 cationic polar part which interacts with the nucleic acids, and a hydrophobic lipid part which promotes cellular penetration. Specific examples of cationic lipids are in particular the monocationic lipids (DOTMA: Lipofectin[®]), some cationic detergents (DDAB), 10 lipopolyamines and in particular dioctadecylamidoglycyl spermine (DOGS) or 5-carboxyspermylamine of palmitoyl-phosphatidylethanolamine (DPPES), whose preparation has been described, for example, in Patent Application EP 394 111. Another lipopolyamine family is represented 15 by the compounds RPR 120531, RPR 122766 or RPR 120535 which are described in Patent Application WO 97/18185 incorporated into the present by way of reference, and are illustrated in Figure 2.

However, up until now, injections into the tissues, and in particular the muscles, were often carried out with naked DNA in order to facilitate its entry into cells, the combination with synthetic vectors leading to complexes with an excessively large size to be incorporated into the cells.

25 This is one of the main problems which the
present invention proposes to solve. Indeed, the agents
for transferring nucleic acids according to the
invention possess the unexpected advantage of

exhibiting a level of transfection *in vivo* into the muscle which is at least equivalent to that obtained with naked DNA. The combination with a compound according to the invention protects the DNA from 5 degradation by nucleases and/or from damage during freeze-drying, which contributes towards significantly improving the stability of the nuclolipid formulations. Furthermore, such a combination allows a slow controlled release of the nucleic acids.

10 Moreover, the agents for transferring nucleic acids according to the present invention are cationic lipids carrying a novel cationic region which confers on the molecules enhanced properties, and in particular a reduced cytotoxicity compared with the prior art 15 cationic vectors. This cationic part is represented more precisely by one or more particular polyamines carrying one or more cyclic amidine functional groups.

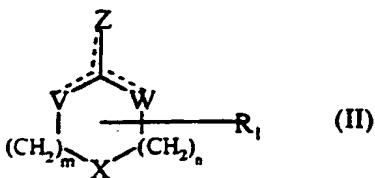
Accordingly, a first subject of the invention relates to a new type of agent for transferring nucleic 20 acids in D, L or DL form, of general formula (I):



for which:

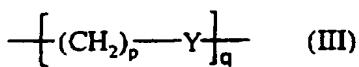
1. CA represents a cycloamidine head and its mesomeric forms of general formula (II):

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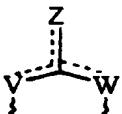
for which:

- m and n are integers, independent of each other, between 0 and 3 inclusive and such that $m+n$ is greater than or equal to 1,
- 5 • R_1 represents a group of general formula (III):



for which p and q are integers, independent of each other, between 0 and 10 inclusive, and Y represents a carbonyl, amine, methylamine or methylene group, it being possible for Y to have different meanings within the different $[(CH_2)_p - Y]$ groups, and it being understood that R_1 may be linked to any atom of the general formula (II),

- 10 • X represents a group NR_2 or CHR_2 , R_2 being either a hydrogen atom or the group R_1 as defined above,

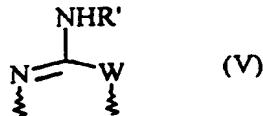
15 • the group  represents:

* 1st case: a group of general formula (IV):



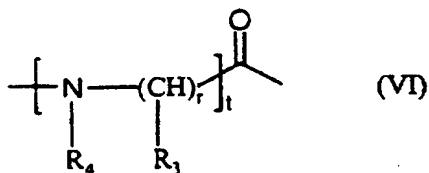
for which W represents CHR''' or NR''' , and R'' and R''' represent, independently of each other, a hydrogen atom, a methyl, or the group R_1 as defined above, it being understood that R'' and R''' cannot be R_1 at the same time, or

* 2nd case: a group of general formula (V):



for which W represents CHR''' or NR''' , and R' and R''' represent, independently of each other, a hydrogen atom, a methyl or the group R_1 as defined above, it being understood that R' and R''' cannot be R_1 at the same time,

2. Rep is absent or is a spacer of general formula (VI):



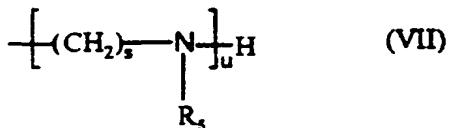
15 whose nitrogen atom is attached to CA, and:

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t is an integer between 0 and 8 inclusive,

r is an integer between 0 and 10 inclusive, it being possible for r to have different meanings within the different $-\text{NR}_4^+ - (\text{CH}_2)_r -$ groups,

5 • R_3 , which may have different meanings within the different $NR_4 - (CH)_x R_3$ groups, represents a hydrogen atom, a methyl group or a group of general formula (VII):



for which u is an integer between 1 and 10 inclusive, s

10 is an integer between 2 and 8 inclusive which may have different meanings within the different $-(CH_2)_s-NR_5$ groups, and R_5 is a hydrogen atom, a group CA as defined above, it being understood that the CA groups are independent of each other and may be different, or a

15 group of general formula (VII), it being understood that the groups of general formula (VII) are independent of each other and may have different meanings.

20 • R_4 is defined in the same way as R_3 , or represents a group CA as defined above, it being understood that the CA groups are independent of each other and may be different, and

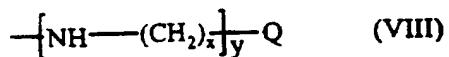
3. R is linked to the carbonyl functional group of the group Rep of general formula (VI), or if

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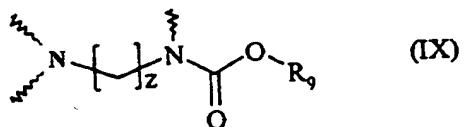
Rep is absent, R is linked directly to CA, and

represents:

- * either a group of formula NR_6R_7 , for which R_6 and R_7 represent, independently of each other, a hydrogen atom
- 5 or an optionally fluorinated, linear or branched, saturated or unsaturated aliphatic radical containing 1 to 22 carbon atoms, with at least one of the two substituents R_6 or R_7 , different from hydrogen and the other containing between 10 and 22 carbon atoms,
- 10 * or a steroid derivative,
- * or a group of general formula (VIII):



for which x is an integer between 1 and 8 inclusive, y is an integer between 1 and 10 inclusive, and either Q represents a group $C(O)NR_6R_7$, for which R_6 and R_7 are as defined above, or Q represents a group $C(O)R_8$ for which R_8 represents a group of formula (IX):



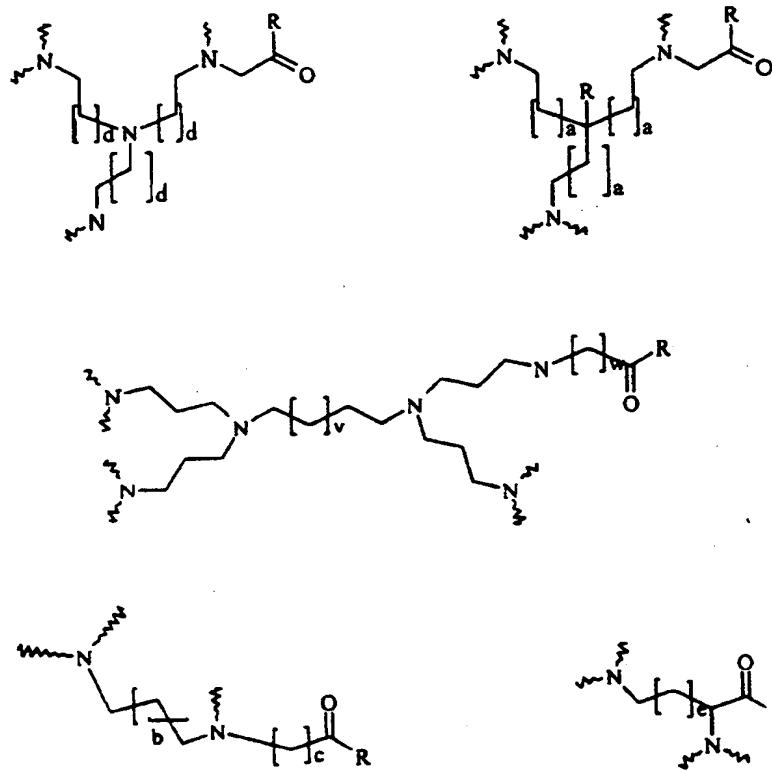
for which z is an integer between 2 and 8 inclusive, and R_9 is an optionally fluorinated, saturated or unsaturated aliphatic radical containing 8 to 22 carbon atoms, or a steroid derivative,

20 or R_8 represents a group $-O-R_9$, for which R_9 is as

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defined above.

In a first variant of the invention, Rep is a spacer with 1, 2 or 3 arms. The following spacers may be mentioned for example:



5 In a second variant of the invention, R_3 and
 R_4 , present in formula (VI), represent hydrogen atoms.

In another variant of the invention, R_4 is a hydrogen atom and R_3 is a group of formula (VII) in which R_5 represents a cycloamidine head CA.

10 Advantageously, the cycloamidine head CA of
formula (II) comprises 5, 6, 7 or 8 members.

Preferably, in formula (III), p and q are

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chosen, independently of each other, from 2, 3 or 4.

As indicated above, the group R contains at least one aliphatic chain or at least one steroid derivative, that is to say more generally at least one 5 hydrophobic segment. For the purposes of the invention, hydrophobic group is understood to mean any group of the lipid type, promoting cellular penetration.

Preferably, the lipophilic group is an aliphatic radical containing 10 to 22 carbon atoms, 10 preferably 12, 14, 16, 17, 18 or 19 carbon atoms, and in particular the groups $(CH_2)_{11}CH_3$, $(CH_2)_{13}CH_3$, $(CH_2)_{15}CH_3$, $(CH_2)_{17}CH_3$, and oleyl (unsaturated chain of 18 carbon atoms).

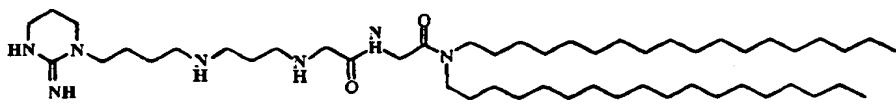
In a specific embodiment, the groups R_5 and R_7 , 15 are identical or different and each represent an optionally fluorinated, linear or branched, saturated or unsaturated aliphatic chain containing 10 to 22 carbon atoms.

When the lipophilic group is a steroid 20 derivative, the latter is advantageously chosen from cholesterol, cholestanol, 3- α -5-cyclo-5- α -cholestan-6- β -ol, cholic acid, cholesteryl formate, chotestanyl formate, 3 α ,5-cyclo-5 α -cholestan-6 β -yl formate, cholesterylamine, 6-(1,5-dimethylhexyl)-3 α ,5a-25 dimethylhexadecahydrocyclopenta[a]cyclopropa[2,3]cyclopenta[1,2-f]naphthalen-10-ylamine, or cholestanylamine.

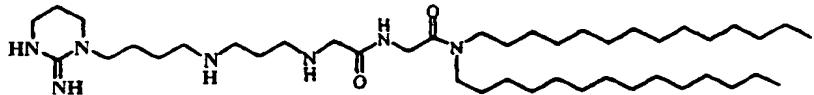
These new agents for transferring nucleic acids of general formula (I) may be provided in the

form of nontoxic and pharmaceutically acceptable salts. These nontoxic salts comprise the salts with inorganic acids (hydrochloric, sulphuric, hydrobromic, phosphoric or nitric acid) or with organic acids (acetic, 5 propionic, succinic, maleic, hydroxymaleic, benzoic, fumaric, methanesulphonic or oxalic acid) or with inorganic bases (sodium hydroxide, potassium hydroxide, lithium hydroxide or calcium hydroxide) or with organic bases (tertiary amines such as triethylamine, 10 piperidine or benzylamine).

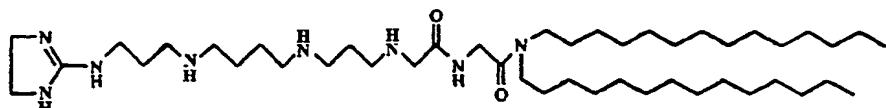
By way of an illustrative example of advantageous agents for transferring nucleic acids according to the invention, the compounds of the following formulae may be mentioned:



15 N-dioctadecylcarbamoylmethyl-2-[3-[4-(2-iminotetrahydropyrimidin-1-yl)butylamino]propylamino]acetamide

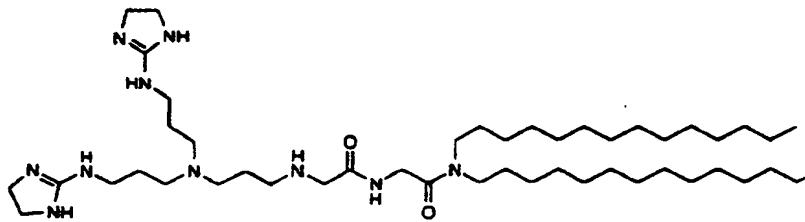


20 N-ditetradecylcarbamoylmethyl-2-[3-[4-(2-iminotetrahydropyrimidin-1-yl)butylamino]propylamino]acetamide



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2-(3-[4-[3-(4,5-dihydro-1H-imidazol-2-ylamino)propylamino]butylamino]-N-ditetradecylcarbamoylmethylacetamide



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2-(3-[bis-[3-(4,5-dihydro-1H-imidazol-2-ylamino)propyl]amino]propylamino)-N-ditetradecylcarbamoylmethylacetamide.

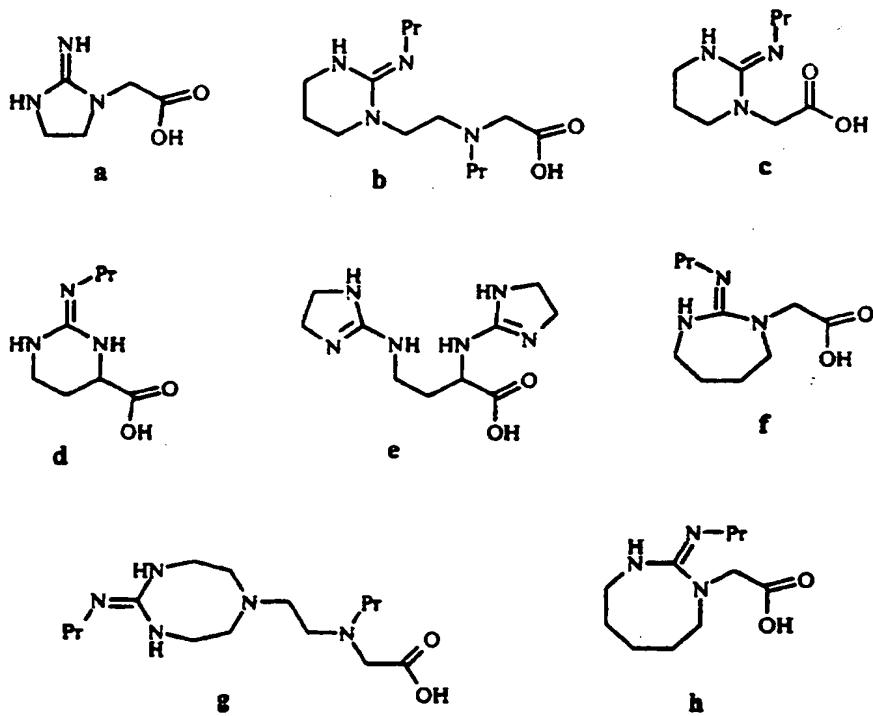
The agents for transferring nucleic acids of the invention may be prepared in various ways. In particular, a first method consists in carrying out a synthesis of "building blocks" carrying the cycloamidine functional group which are then grafted onto lipids equipped with spacers. This method has the advantage of providing access to a large number of products. Another method corresponds to the synthesis of lipopolyamines, the cyclization into cycloamidine heads being carried out in a second stage.

For the purposes of the invention, "building

blocks" are understood to mean any functional segment of a molecule. For example, the cycloamidine head CA as defined in the general formula (II), Rep or R each constitute building blocks for the purposes of the 5 invention.

Building blocks carrying the cycloamidine functional group(s) and which can be directly coupled are for example:

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10 The cyclization of the amidine heads according to the second mode of synthesis described above may, for example, be carried out by the reaction between one and/or more primary amines situated in a molecule of interest and reagents such as O-methylisourea hydrogen sulphate (ref.: J. Med. Chem.

38 (1995) 16, 3053-3061) or S-methylisothiourea hemisulphate (ref.: Int. J. Pept. Prot. Res. 40 (1992), 119-126). Other methods and/or other reagents known to persons skilled in the art may of course be used.

5 Another subject of the invention relates to a composition comprising an agent for transferring nucleic acids as defined above, and a nucleic acid. Preferably, the transfer agent and the nucleic acid are present in quantities such that the ratio R of the
10 positive charges of the compound to the negative charges of the nucleic acid is between 0.1 and 50, preferably between 0.1 and 20. This ratio can be easily adjusted by persons skilled in the art according to the compound used, the nucleic acid and the desired
15 applications (in particular the type of cells to be transfected).

For the purposes of the invention, "nucleic acid" is understood to mean both a deoxyribonucleic acid and a ribonucleic acid. They may be natural or
20 artificial sequences, and in particular genomic DNA (gDNA), complementary DNA (cDNA), messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), hybrid sequences or synthetic or semisynthetic sequences, oligonucleotides which are modified or otherwise. These
25 nucleic acids may be of human, animal, plant, bacterial or viral origin and the like. They may be obtained by any technique known to persons skilled in the art, and in particular by the screening of libraries, by

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chemical synthesis or by mixed methods including the chemical or enzymatic modification of sequences obtained by the screening of libraries. They may be chemically modified.

5 As regards more particularly deoxyribonucleic acids, they may be single- or double-stranded, as well as short oligonucleotides or longer sequences. In particular, the nucleic acids advantageously consist of plasmids, vectors, episomes, expression cassettes and
10 the like. These deoxyribonucleic acids may carry a replication origin which is functional or otherwise in the target cell, one or more marker genes, sequences for regulating transcription or replication, genes of therapeutic interest, anti-sense sequences which are
15 modified or otherwise, regions for binding to other cellular components, and the like.

Preferably, the nucleic acid comprises an expression cassette consisting of one or more genes of interest under the control of one or more promoters and
20 of a transcriptional terminator which are active in the target cells.

For the purposes of the invention, gene of therapeutic interest is understood to mean in particular any gene encoding a protein product having a
25 therapeutic effect. The protein product thus encoded may in particular be a protein or a peptide. This protein product may be exogenous, homologous or

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endogenous in relation to the target cell, that is to say a product which is normally expressed in the target cell when the latter has no pathological condition. In this case, the expression of a protein makes it

5 possible, for example, to palliate an insufficient expression in the cell or the expression of a protein which is inactive or weakly active because of a modification, or to overexpress the said protein. The gene of therapeutic interest may also encode a mutant

10 of a cellular protein, having increased stability, a modified activity and the like. The protein product may also be heterologous in relation to the target cell. In this case, an expressed protein may, for example, supplement or provide an activity which is deficient in

15 the cell, allowing it to combat a pathological condition, or to stimulate an immune response.

Among the therapeutic products for the purposes of the present invention, there may be mentioned more particularly enzymes, blood derivatives, hormones, lymphokines: interleukins, interferons, TNF, and the like (FR 92/03120), growth factors, neuro-transmitters or their precursors or synthesis enzymes, trophic factors (BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5, HARP/pleiotrophin and the like),

20 apolipoproteins (ApoAI, ApoAIV, ApoE, and the like, FR 93/05125), dystrophin or a minidystrophin (FR 91/11947), the CFTR protein associated with cystic fibrosis, tumour suppressor genes (p53, Rb, Rap1A, DCC,

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5 k-rev, and the like FR 93/04745), genes encoding factors involved in coagulation (factors VII, VIII, IX), the genes involved in DNA repair, suicide genes (thymidine kinase, cytosine deaminase), the genes for haemoglobin or other protein carriers, metabolic enzymes, catabolic enzymes and the like.

The nucleic acid of therapeutic interest may also be a gene or an anti-sense sequence, whose expression in the target cell makes it possible to control the expression of genes or the transcription of cellular mRNAs. Such sequences can, for example, be transcribed in the target cell into RNAs which are complementary to cellular mRNAs and thus block their translation to protein, according to the technique described in Patent EP 140 308. The therapeutic genes also comprise the sequences encoding ribozymes, which are capable of selectively destroying target RNAs (EP 321 201).

As indicated above, the nucleic acid may also
20 comprise one or more genes encoding an antigenic
peptide, which is capable of generating an immune
response in humans or in animals. In this specific
embodiment, the invention allows the production of
vaccines or the carrying out of immunotherapeutic
25 treatments applied to humans or to animals, in
particular against microorganisms, viruses or cancers.
They may be in particular antigenic peptides specific
for the Epstein-Barr virus, the HIV virus, the

hepatitis B virus (EP 185 573), the pseudo-rabies virus, the syncitia-forming virus, other viruses, or antigenic peptides specific for tumours (EP 259 212).

Preferably, the nucleic acid also comprises sequences allowing the expression of the gene of therapeutic interest and/or the gene encoding the antigenic peptide in the desired cell or organ. They may be sequences which are naturally responsible for the expression of the gene considered when these sequences are capable of functioning in the infected cell. They may also be sequences of different origin (responsible for the expression of other proteins, or even synthetic). In particular, they may be promoter sequences of eukaryotic or viral genes. For example, they may be promoter sequences derived from the genome of the cell which it is desired to infect. Likewise, they may be promoter sequences derived from the genome of a virus. In this regard, there may be mentioned, for example, the promoters of the E1A, MLP, CMV and RSV genes, and the like. In addition, these expression sequences may be modified by the addition of activating or regulatory sequences, and the like. The promoter may also be inducible or repressible.

Moreover, the nucleic acid may also comprise, in particular upstream of the gene of therapeutic interest, a signal sequence directing the therapeutic product synthesized in the secretory pathways of the target cell. This signal sequence may be the natural

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signal sequence of the therapeutic product, but it may also be any other functional signal sequence, or an artificial signal sequence. The nucleic acid may also comprise a signal sequence directing the synthesized 5 therapeutic product towards a particular compartment of the cell.

The compositions may, in addition, comprise adjuvants capable of combining with the transfer agent/nucleic acid complexes and of improving its 10 transfecting power. In another embodiment, the present invention therefore relates to compositions comprising a nucleic acid, an agent for transferring nucleic acids as defined above and an adjuvant capable of combining with the transfer agent/nucleic acid complexes and of 15 improving the transfecting power thereof. The presence of this type of adjuvant (lipids, peptides or proteins for example) may make it possible advantageously to increase the transfecting power of the compounds.

In this regard, the compositions according to 20 the invention may comprise, as adjuvant, one or more neutral lipids. Such compositions are particularly advantageous, in particular when the ratio R is low. The Applicant has indeed shown that the addition of a neutral lipid makes it possible to improve the 25 formation of nucleolipid particles and to promote the penetration of the particle into the cell by destabilizing its membrane...

More preferably, the neutral lipids used

within the framework of the present invention are lipids containing two fatty chains. In a particularly advantageous manner, natural or synthetic lipids which are zwitterionic or lacking ionic charge under 5 physiological conditions are used. They may be chosen more particularly from dioleoylphosphatidylethanolamine (DOPE), oleoylpalmitoylphosphatidylethanolamine (POPE), di-stearoyl, -palmitoyl, -mirystoylphosphatidylethanol- amines as well as their derivatives which are 10 N-methylated 1 to 3 times, phosphatidylglycerols, diacylglycerols, glycosyldiacylglycerols, cerebrosides (such as in particular galactocerebrosides), sphingolipids (such as in particular sphingomyelins) or asialogangliosides (such as in particular asialoGM1 and 15 GM2).

These different lipids may be obtained either by synthesis or by extraction from organs (for example the brain) or from eggs, by conventional techniques well known to persons skilled in the art. In 20 particular, the extraction of the natural lipids may be carried out by means of organic solvents (see also Lehninger, Biochemistry).

More recently, the Applicant has demonstrated that it was also particularly advantageous to use, as 25 adjuvant, a compound involved directly or otherwise in the condensation of the said nucleic acid (WO 96/25508). The presence of such a compound in a composition according to the invention makes it

possible to reduce the quantity of transfecting agent, with the beneficial consequences resulting therefrom from the toxicological point of view, without any damaging effect on the transfecting activity. Compound 5 involved in the condensation of the nucleic acid is intended to define a compound which compacts, directly or otherwise, the nucleic acid. More precisely, this compound may either act directly at the level of the nucleic acid to be transfected, or may be involved at 10 the level of an additional compound which is directly involved in the condensation of this nucleic acid. Preferably, it acts directly at the level of the nucleic acid. For example, the precompacting agent may be any polycation, for example polylysine. According to 15 a preferred embodiment, this agent which is involved in the condensation of the nucleic acid is derived as a whole or in part, from a protamine, a histone or a nucleolin and/or from one of their derivatives. Such an agent may also consist, as a whole or in part, of 20 peptide units (KTPKKAKKP) and/or (ATPAKKAA), it being possible for the number of units to vary between 2 and 10. In the structure of the compound according to the invention, these units may be repeated continuously or otherwise. They may thus be separated by linkages of a 25 biochemical nature, for example one or more amino acids, or of a chemical nature.

Preferably, the compositions of the invention comprise from 0.01 to 20 equivalents of adjuvant for

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one equivalent of nucleic acids in mol/mol and, more preferably, from 0.5 to 5.

In a particularly advantageous embodiment, the compositions of the present invention comprise, in 5 addition, a targeting element which makes it possible to orient the transfer of the nucleic acid. This targeting element may be an extracellular targeting element which makes it possible to orient the transfer of DNA towards certain cell types or certain desired 10 tissues (tumour cells, hepatic cells, haematopoietic cells and the like). It may also be an intracellular targeting element which makes it possible to orient the transfer of the nucleic acid towards certain preferred cellular compartments (mitochondria, nucleus and the 15 like). The targeting element may be linked to the nucleic acid transfer agent according to the invention or also to the nucleic acid as specified above.

Among the targeting elements which may be used within the framework of the invention, there may 20 be mentioned sugars, peptides, proteins, oligonucleotides, lipids, neuromediators, hormones, vitamins or derivatives thereof. Preferably, they are sugars, peptides or proteins such as antibodies or antibody fragments, ligands of cell receptors or 25 fragments thereof, receptors or receptor fragments, and the like. In particular, they may be ligands of growth factor receptors, cytokine receptors, cellular lectin-type receptors, or RGD sequence-containing ligands with

an affinity for the receptors for adhesion proteins such as the integrins. There may also be mentioned the receptors for transferrin, HDLs and LDLs, or the folate transporter. The targeting element may also be a sugar which makes it possible to target lectins such as the receptors for asialoglycoproteins or for sialydes such as the sialyde Lewis X, or alternatively an Fab fragment of antibodies, or a single-chain antibody (ScFv).

20 According to another variant, the compositions of the invention may also incorporate at least one nonionic surfactant in a sufficient quantity to stabilize the size of the particles of nucleolipid complexes. The introduction of nonionic surfactants 25 prevents the formation of aggregates, which makes the composition more suitable for an *in vivo* administration. The compositions according to the invention incorporating such surfactants have an

advantage from the point of view of safety. They also have an additional advantage in the sense that they reduce the risk of interference with other proteins, given the reduction in the overall charge of the 5 compositions of nucleolipid complexes.

The surfactants advantageously consist of at least one hydrophobic segment, and at least one hydrophilic segment. Preferably, the hydrophobic segment is chosen from aliphatic chains, polyoxy-10 alklenes, alkylidene polyesters, polyethylene glycols with a benzyl polyether head and cholesterol, and the hydrophilic segment is advantageously chosen from polyoxyalklenes, polyvinyl alcohols, polyvinyl-pyrrolidones, or saccharides. Such nonionic surfactants 15 have been described in application PCT/FR 98/00222.

The subject of the invention is also the use of the compounds as defined above for the transfer of nucleic acids (and more particularly of polyanions) into cells, *in vitro*, *ex vivo* or *in vivo*. Such a use is 20 particularly advantageous because the agents for transferring nucleic acids according to the invention have a reduced cytotoxicity compared with the cationic lipids of the prior art. The applicant has in particular demonstrated that at very high charge ratios 25 which usually cause the death of the animals following the transfection, no apparent cytotoxicity was detected.

For uses *in vivo*, for example in therapy or

for studying the regulation of genes or the creation of animal models of pathological conditions, the compositions according to the invention can be formulated for administration by the topical, cutaneous, oral, rectal, vaginal, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal, intratracheal or intraperitoneal route, and the like. Preferably, the compositions of the invention contain a vehicle which is pharmaceutically acceptable for an injectable formulation, in particular a direct injection into the desired organ, or for administration by the topical route (on the skin and/or the mucous membrane). They may be in particular isotonic sterile solutions, or dry, in particular freeze-dried, compositions which, upon addition, depending on the case, of sterilized water or of physiological saline, allow the constitution of injectable solutions. The nucleic acid doses used for the injection as well as the number of administrations may be adapted according to various parameters, and in particular according to the mode of administration used, the relevant pathological condition, the gene to be expressed, or the desired duration of treatment. As regards more particularly the mode of administration, it may be either a direct injection into the tissues, for example at the level of the tumours, or the circulatory system, or a treatment of cells in culture followed by their reimplantation *in vivo* by injection or transplantation.

16 0 0 0 8 5 8 8 4 5 " 0 9 5 1 8 8 9 8 8

The relevant tissues within the framework of the present invention are, for example, the muscles, skin, brain, lungs, liver, spleen, bone marrow, thymus, heart, lymph, blood, bones, cartilages, pancreas, 5 kidneys, bladder, stomach, intestines, testicles, ovaries, rectum, nervous system, eyes, glands, connective tissues, and the like. Advantageously, the transfected tissues are the muscles.

The invention relates, in addition, to a 10 method of transferring nucleic acids into cells comprising the following steps:

- (1) bringing the nucleic acid into contact with a compound as defined above, to form a nucleolipid complex, and
- 15 (2) bringing the cells into contact with the complex formed in (1).

The cells may be brought into contact with the complex by incubating the cells with the said complex (for uses *in vitro* or *ex vivo*), or by injecting 20 the complex into an organism (for uses *in vivo*). The incubation is carried out preferably in the presence, for example, of 0.01 to 1000 µg of nucleic acid per 10^6 cells. For administration *in vivo*, nucleic acid doses of between 0.01 and 10 mg may for example be used.

25 In the case where the compositions of the invention contain, in addition, one or more adjuvants as defined above, the adjuvant(s) is (are) previously mixed with the lipid according to the invention or with

the nucleic acid.

The present invention thus provides a particularly advantageous method for transferring nucleic acids *in vivo*, in particular for the treatment of diseases, comprising the *in vivo* or *in vitro* administration of a nucleic acid encoding a protein or which can be transcribed into a nucleic acid capable of correcting the said disease, the said nucleic acid being combined with a compound of the general formula 10 (I) under the conditions defined above. More particularly, this method is applicable to diseases resulting from a deficiency in a protein or nucleic product, the administered nucleic acid encoding the said protein product or being transcribed into a 15 nucleic product or alternatively constituting the said nucleic product.

The invention extends to any use of a nucleic acid transfer agent according to the invention for the *in vivo*, *ex vivo* or *in vitro* transfection of cells.

20 The nucleic acid transfer agents of the invention can be used in particular for transferring nucleic acids into primary cells or into established lines. They may be fibroblast cells, muscle cells, nerve cells (neurons, astrocytes, glial cells), hepatic 25 cells, haematopoietic cells (lymphocytes, CD34, dendritic cells, and the like), epithelial cells and the like, in differentiated or pluripotent form (precursors).

In addition to the preceding arrangements, the present invention also comprises other characteristics and advantages which will emerge from the examples and figures below, which should be considered as illustrating the invention without limiting its scope.

FIGURES

Figure 1: Structure of the agents for transferring nucleic acids 1, 2, 3 and 4 according to the invention.

Figure 2: Structure of the synthetic vectors

5 RPR 120535, RPR 121650, RPR 120531 and RPR 122766
(which are described in Patent Application WO 97/18185
incorporated into the present by reference).

Figure 3: Schematic representation of the plasmid
pXL2774.

10 Figures 4 and 5: Phase diagram for the transfer agent/
DNA complexes. The binding of the lipid to DNA was
determined by following the decrease in the
fluorescence (in %, 100% being the fluorescence of the
naked DNA) of ethidium bromide (EtBr) (symbol ●, solid
15 line), as described according to the y-axis situated on
the right. The size of the particles of complexes
(in nm) is indicated on the y-axis situated on the
left. The x-axis represents the transfer agents/DNA
charge ratio. The size of the nucleolipid complexes
20 without co-lipid is represented by the symbol ■ as a
solid line. The size of the nucleolipid complexes
containing 25% cholesterol is represented by the symbol
□ as a discontinuous line. The size of the nucleolipid
complexes containing 40% DOPE is represented by the
25 symbol ◆ as a discontinuous line. The method does not
make it possible to determine the size of particles
above 3 μ m.

Figure 6: Activity for in vitro gene transfer into HeLa

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cells of the complexes containing compound 1 according to the present invention without co-lipid (dark-shaded middle bar), with 25% cholesterol (medium-shaded left-hand bar), and with 40 mol % of DOPE (light-shaded right-hand bar), compared with naked DNA. Only the complexes in which the DNA is completely saturated with lipid and whose size is between 100 nm and 300 nm were used.

Figure 7: *In vivo* gene transfer activity, after direct injection into the muscle, of the complexes containing compound 1 according to the present invention or the compound [lacuna] medium-shaded), and with 40 mol % of DOPE (light-shaded bar), compared with naked DNA. Only the complexes in which the DNA is completely saturated with lipid and whose size is between 100 nm and 300 nm were used.

Figure 8: The importance of the invention is illustrated by comparing the gene transfer activity of two different lipids, compound 1 according to the invention and RPR 120531, and naked DNA via two routes of administration: by the intravenous (IV) route and by the intramuscular (IM) route. Only the complexes in which the DNA is completely saturated with lipid and whose size is between 100 nm and 300 nm were used.

25 ABBREVIATIONS AND SYMBOLS

AcOEt:	ethyl acetate
BOC:	t-butoxycarbonyl

	BOP:	benzotriazol-1- yloxytris(dimethylamino)phosphonium hexafluorophosphate
	CH_2Cl_2 :	dichloromethane
5	CHCl_3 :	chloroform
	DCC:	dicyclohexylcarbodiimide
	DCE:	1,2-dichloroethane
	DCU:	dicyclohexylurea
	DIEA:	N-ethyldiisopropylamine
10	DMAP:	4-dimethylaminopyridine
	DMF:	dimethylformamide
	DMSO:	dimethyl sulphoxide
	DODA:	dioctadecylamine
	EP:	petroleum ether
15	EtBr :	ethyldium bromide
	EtOH :	ethanol
	KHSO_4 :	potassium sulphate
	MeOH :	methanol
	MeCN:	acetonitrile
20	MgSO_4 :	magnesium sulphate
	NaCl:	sodium chloride
	NaHCO_3 :	sodium carbonate
	NaOH:	sodium hydroxide
	NEt_3 :	triethylamine
25	Rf:	retardation factor
	TEA:	triethylamine
	TFA:	trifluoroacetic acid
	THF:	tetrahydrofuran

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TMS:	tetramethylsilane
UV:	ultraviolet
SPPS:	solid phase peptide synthesis
TLC:	thin-layer chromatography
5 HPLC:	high-performance liquid chromatography
Z:	benzyloxycarbonyl
ClZ:	p-chlorobenzyloxycarbonyl

MATERIALS AND METHODS

A\ MATERIALS

10 - The starting polyamine amino acids (or derivatives thereof) are commercially available. This is the case, for example, for: N-(3-aminopropyl)glycine, N-(2-cyanoethyl)glycine and 2,4-diaminobutyric acid, or may be synthesized by
15 conventional methods.

- The cyclic isothioureas are also commercial products, such as for example 2-methylthio-2-imidazoline hydroiodide, or may be synthesized by conventional methods known to persons skilled in the
20 art.

- The lipid amines are commercial products or else synthesized from the corresponding amines and aldehydes by alkylative reduction.

- The products triethylamine,
25 trifluoroacetic, BOP, DMAP, benzyl chloroformate, di-tert-butyl dicarbonate are commercial products. The NaCl and NaHCO₃ solutions are saturated; the KHSO₄

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solution is 0.5 M.

B\METHODS

1) Physical measurements

5 The proton NMR spectra were recorded on
Bruker 400 and 600 MHz spectrometers.

The mass spectra were taken on an API-MS/III.

2) Methods of purification and analysis

a) Direct-phase chromatography conditions

10 - The thin-layer chromatographies (TLC) were
carried out on 0.2 mm thick Merck silica gel plates.

They are developed either under UV (254 nm),
with ninhydrin, by spraying (light spray) an ethanolic
solution of ninhydrin (40 mg/100 ml of EtOH) in order
to reveal the amines or the amides by heating to 150°C,
15 with fluorescamine, by spraying a solution (40 mg/
100 ml of acetone) in order to reveal the primary
amines, with bromocresol green, by spraying a solution
(0.1% in 2-propanol) in order to reveal the acids, with
vanillin by spraying (light spray) an ethanolic
20 solution of vanillin (3%) with 3% H₂SO₄ followed by
heating to 120°C, or with iodine by covering the plate
with iodine powder.

25 - The column chromatographies were carried
out on a Merck 60 silica gel having a particle size of
0.063-0.200 mm.

b) Preparative HPLC purification conditions

The equipment used is a set for liquid-phase

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chromatography in gradient mode, allowing UV detection.

This preparative chain is composed of the following components:

Pump A: GILSON model 305 equipped with a 50 SC head.

5 Pump B: GILSON model 303 equipped with a 50 SC head.

Injection loop: 5 ml.

Pressure module: GILSON model 806.

Mixer: GILSON model 811 C equipped with a 23 ml head.

UV detector: GILSON model 119, equipped with a

10 preparative cell.

Fraction collector: GILSON model 202 equipped with No.

21 racks and with a glass tube of 10 ml.

Integrator: SHIMADZU model C-R6A.

Column: Column C4 (10 mm) made of stainless steel 25 cm
15 long and 2.2 cm in diameter, marketed by VYDAC model
214 TP 1022.

20 The solution of product to be purified is
loaded onto the column by means of the injection loop,
the eluate is recovered in fractions of one tube in 30
seconds. The detector is set at the wavelengths of
220 nm and 254 nm.

The mobile phases are defined as follows:

<u>Solvent A</u>		<u>Solvent B</u>	
Demineralized water	2500 ml	Acetonitrile	2500 ml
		for HPLC	
Trifluoroacetic acid	2 ml	Trifluoro-	2.5 ml
		acetic acid	

Gradient:

5	Time in min	% of solvent A	% of solvent B	Flow rate in ml/min
	0	90	10	18
	10	90	10	18
	110	0	100	18
	120	0	100	18

10 c) Analytical chromatography techniques

- The HPLC (high performance liquid chromatography) analyses were carried out on a Merck-Hitachi apparatus equipped with a HITACHI D 2500 integrator-calculator, an autosampler AS-2000A, an Intelligent Pump L-6200A, a visible UV detector L-4000 with an adjustable wavelength set at 220 nm.

The columns for the analytical separations are Browlee columns made of stainless steel 3 cm long and 0.46 cm in diameter, marketed by APPLIED BIOSYSTEM.

20 The stationary phase consists of Aquapore

Butyl 7 micron. The mobile phases are water (with TFA) and acetonitrile (with TFA). The injections are 20 μ l of a solution of about 1 mg/ml in a 100 μ l loop valve. The flow rate for the analyses is adjusted between 1 ml/min and 4 ml/min. The pressure is about 180 bar. The separation conditions are summarized below:

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<u>Solvent A</u>	<u>Solvent B</u>
Demineralized water	2500 ml Acetonitrile 2500 ml for HPLC
Trifluoroacetic acid	2 ml Trifluoro- 2.5 ml acetic acid

Gradient:

5	Time in min	% of solvent A	% of solvent B	Flow rate in ml/min
	0	60	40	1
	3	60	40	1
	20	0	100	1
	35	0	100	1
10	35.1	60	40	4
	36.1	60	40	4
	36.2	60	40	2
	44	60	40	2

C\GENERAL PROCEDURE15 a) Method A: Coupling of an organic acid with an amine

The organic acid (10 mmol) and the amine (10 mmol) are introduced into a 250 ml round-bottomed flask, CH_2Cl_2 (100 ml) is added and the mixture is stirred until complete dissolution is obtained. The

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products DIEA (30 mmol) and BOP (11 mmol) are then added. The [lacuna] When the reaction is complete (monitored by TLC and/or HPLC), the dichloromethane is evaporated off and the solid obtained is taken up in 5 ethyl acetate (300 ml). The organic phase is washed with a solution of KHSO_4 (four times 100 ml), NaHCO_3 (four times 100 ml), and NaCl (four times 100 ml). The organic phase is dried over MgSO_4 , filtered and evaporated under vacuum. The products are analysed by 10 TLC, NMR and MS, and are used without further purifications. The yields are of the order of 90%.

b) Method B: Cleavage of the Boc groups

The amine-containing product protected by Boc groups (1 mmol) is introduced into a round-bottomed 15 flask equipped with a magnetic bar. 30 ml of TFA at 4°C are added and then the solution is stirred for one hour. When the reaction is complete (monitored by TLC and/or HPLC), the TFA is evaporated under vacuum and then the product is dried by coevaporation with three 20 times 30 ml of ethyl ether.

c) Method C: Cleavage of the Z groups

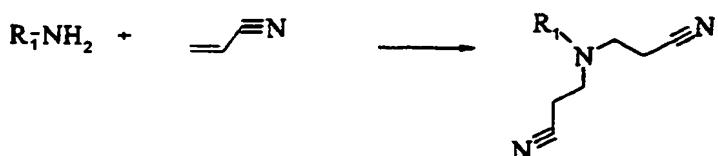
The products containing the Z or ClZ groups are introduced into a round-bottomed flask equipped 25 with a magnetic bar and dissolved in 10 ml of MeOH/g of product. Palladium on carbon (10%, 1 g/g of product) and ammonium formate (1 g/g of product) are added at room temperature. The hydrogenolysis is monitored by HPLC. After two hours, the reaction is complete, the

5 mixture is filtered and the filter is washed with three times 10 ml of MeOH/g of product. Double-distilled water is added and the solution is frozen and freeze-dried, or alternatively the filtrate is concentrated to dryness and the solid is taken up in ethyl acetate (300 ml). The organic phase is washed with NaHCO₃ (twice 100 ml), and NaCl (twice 100 ml), and it is then dried under MgSO₄, filtered and evaporated under vacuum. The products are analysed by HPLC and are used without 10 further purifications. The yields are of the order of 90%.

d) Method D: Preparation of the Boc amino acids or Z amino acids

The amino acid (0.1 mol/amine) is solubilized
15 in 1 N sodium hydroxide (200 ml/amine) and dioxane
(200 ml). The solution is stirred in an ice bath and
then a solution of (Boc)₂O or ClZ (0.14 mol/amine) in
200 ml of dioxane is added dropwise. The pH is kept at
a value greater than 9. The mixture is then stirred at
20 room temperature overnight. The dioxane is evaporated
under vacuum and then the mixture is acidified to pH 3
with the aid of a solution of KHSO₄. The insoluble
matter is extracted with ethyl acetate (three times
100 ml) and then washed with a solution of NaCl (twice
25 100 ml). The organic phase is dried over MgSO₄, filtered
and evaporated under vacuum. The products are analysed
by TLC and/or HPLC.

e) Method E: Cyanoethylation and dicyanoethylation of an amino acid



Cyanoethylation:

The amino acid (0.05 mol) and NaOH (0.1 mol) 5 are solubilized in 150 ml of water in a round-bottomed flask. The solution is cooled in an ice bath. Acrylonitrile (0.05 mol) is slowly added, with vigorous stirring while keeping the temperature of the mass below 20°C. The reaction mixture is kept overnight at 10 room temperature.

The solvent is evaporated under vacuum and then the mixture is acidified to pH 3 with a solution of KHSO_4 . The insoluble matter is extracted with ethyl acetate (three times 200 ml) and then washed with a 15 solution of NaCl (twice 100 ml). The organic phase is dried over MgSO_4 and then filtered and evaporated under vacuum.

The crude matter is optionally purified on a silica column.

20 Dicyanoethylation:

The procedure is identical to that described

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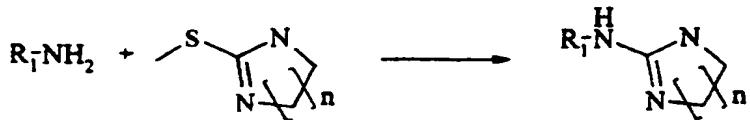
above, with 0.12 mol of acrylonitrile. A step of keeping the mixture at 50°C for two hours is added after the step at room temperature overnight.

f) Method F: Reduction of the nitrile of a cyanoamino

5 acid

The cyanoamino acid (50 mmol) is introduced into a 1-litre stainless steel autoclave. A solution of 10 ml of ethanol (95%) and of 3.3 g of NaOH (80 mol) is prepared at the same time in a beaker. When the sodium hydroxide has dissolved, this solution is introduced into the autoclave. A nitrogen stream is passed through the autoclave and 2 ml of Raney nickel on carbon are introduced. The autoclave is closed. The initial hydrogenation pressure is 52 bar and it decreases to 15 48.5 bar overnight at room temperature. The suspension is filtered on paper, the filter is washed with ethanol (four times 25 ml), and the filtrates are concentrated to dryness under vacuum. A white solid is obtained which is used without further purifications after TLC 20 analysis.

g) Method G: Introduction of a guanidine ring from cyclic isothiourea hydroiodide

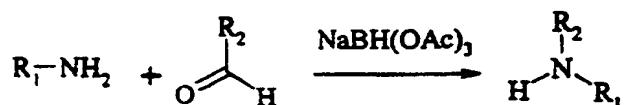


The product containing the primary amine to be modified (1 mmol/amine) is solubilized in CH_2Cl_2 .

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(10 ml) and then cyclic isothiourea hydroiodide (1.2 mmol/amine) and TEA (1.3 mmol/amine) are added. The mixture is stirred at room temperature until the evolution of methyl sulphide stops. At the end of the 5 reaction (monitored by HPLC), the CH_2Cl_2 is evaporated under vacuum.

h) Method H: Synthesis of dilipid amine



The lipid amine (0.02 mol) and the lipid aldehyde (0.02 mol) are solubilized in 200 ml of DCE in 10 a round-bottomed flask. The solution is stirred, and then sodium triacetoxyborohydride (0.028 mol) is added. The mixture is kept stirring overnight. 200 ml of CHCl_3 are poured in and then the solution is washed with NaHCO_3 (twice 100 ml) and NaCl (twice 100 ml). The 15 organic phase is dried over MgSO_4 and then filtered and evaporated under vacuum. The products are analysed by TLC and then purified on a silica column ($\text{CHCl}_3/\text{MeOH}$). The yields are close to 50%.

EXAMPLES

20 **A\SYNTHESSES OF THE TRANSFECTION AGENTS**

Example 1: Synthesis of compound 1 (N-dioctadecyl-carbamoylmethyl-2-{3-[4-(2-iminotetrahydropyrimidin-1-yl)butylamino]propyl- amino}acetamide) from the compound RPR 120535A (whose preparation has been

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described in patent application WO 97/18185 and whose structure is represented in Figure 2).

0.784 mmol of RPR 120535A is dissolved in 25 ml of methanol in a round-bottomed flask equipped with a magnetic bar, and 10.21 mmol of triethylamine are added. Next a solution of O-methylisourea and sulphuric acid H_2SO_4 (1.173 mmol) in water (9 ml) is slowly poured (5 minutes) over the mixture. The mixture is kept at 50°C in an oil bath for twenty hours.

10 Next, the mixture is concentrated to dryness
in a rotary evaporator. The dry extract is solubilized
with a solution of water (4 ml), ethanol (4 ml) and
trifluoroacetic acid (1 ml). This solution is injected
in two portions in preparative HPLC.

15 The fractions of interest (determined by analytical HPLC) are grouped together, frozen and freeze-dried. 194 mg (0.163 mmol) of salified product are thus obtained.

Yield: 20.8%

20 HPLC_{analytical}: Rt = 15.99 minutes.

¹H NMR spectrum (400 MHz, (CD₃)₂SO-d₆, δ in ppm) : 0.88 (t, J = 6.5 Hz, 6H : CH₃ of the fatty chains) ; 1.24 (mt, 60H : central CH₂ of the fatty chains) ; from 1.35 to 1.70 (mt, 4H : 1 CH₂ of each fatty chain) ; 1.57 (mt, 25 4H : central (CH₂)₂ of the butyl) ; 1.88 and 1.96 (2 mts, 2H each : central CH₂ of the propyl and central CH₂ of the ring) ; from 2.85 to 3.35 (2 mts, 16H in total : the 8 NCH₂) ; 3.81 (broad s, 2H : NCH₂CON) ;

4.03 (d, $J = 5$ Hz, 2H : CONCH_2CON of the glycyl) ; 7.25 and 7.84 (s and broad s respectively, 1H each : the 2 NH of the ring) ; 8.61 (t, $J = 5.5$ Hz, 1H : NHCO) ; 8.70 and 9.02 (2 unres. comp., 1H each : the 2 NH).

5 $\text{MH}^+ = 846$

Example 2: Synthesis of compound 2 (N-ditetradecyl-carbamoylmethyl-2-{3-[4-(2-iminotetrahydropyrimidin-1-yl)butylamino]propylamino}acetamide) from the compound RPR 122766A (whose preparation has been described in 10 patent application WO 97/18185 and whose structure is represented in Figure 2).

15 1.036 mmol of RPR 122766A are dissolved in 30 ml of methanol in a round-bottomed flask equipped with a magnetic bar, and 13.13 mmol of triethylamine are added. Next, a solution of O-methylisourea and sulphuric acid H_2SO_4 (1.554 mmol) in water (9 ml) is slowly poured (5 minutes) over the mixture. The mixture is kept at 50°C in an oil bath for about twenty hours. Next, the mixture is concentrated to dryness in a 20 rotary evaporator. The dry extract is solubilized with a solution of water (3 ml), ethanol (2 ml) and trifluoroacetic acid (0.5 ml). This solution is injected in preparative HPLC.

25 The fractions of interest (determined by analytical HPLC) are grouped together, frozen and freeze-dried. 218 mg (0.2022 mmol) of salified product are finally obtained.

Yield: Y = 19.5%

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HPLC_{analytical}: Rt = 10.76 minutes.

¹H NMR spectrum (400 MHz, (CD₃)₂SO-d₆, δ in ppm) : 0.88 (t, J = 7 Hz, 6H : CH₃ of the fatty chains) ; from 1.15 to 1.40 (mt, 44H : central (CH₂)₁₁ of the fatty chains) 5 ; 1.45 and from 1.50 to 1.65 (2 mts, 2H each : 1 CH₂ of each fatty chain) ; 1.59 (mt, 4H : the two central CH₂ of the butyl) ; 1.91 and 1.97 (2 mts, 2H each ; central CH₂ of the propyls) ; from 2.85 to 3.10 (mt, 10H : the 2 NCH₂ of the butyl - the 2 NCH₂ of one of the 2 propyls - 10 and 1 of the 2 NCH₂ of the other propyl) ; 3.23 and from 3.30 to 3.50 (2 mts, 5H and 1H respectively : the other NCH₂ of the other propyl and NCH₂ of the fatty chains) ; 15 3.79 (unres. comp., 2H : NCH₂CON) ; 4.03 (d, J = 5 Hz, 2H : CONCH₂CON of the glycyl) ; 7.27 and from 8.40 to 9.30 (broad s and unres. comp. respectively, 2H and 4H : NH₂⁺ CF₃COO⁻, NH⁺ CF₃COO⁻ and =NH) ; 7.88 and 8.61 (s and broad s respectively, 1H each : NHC=N and CONH respectively).

MH⁺ = 734

20 Example 3: Synthesis of compound 3 (2-(3-{4-[3-(4,5-dihydro-1H-imidazol-2-ylamino)propylamino]butylamino}-propylamino)-N-ditetradecylcarbamoylmethylacetamide) from the compound RPR 122766A (whose preparation has been described in patent application WO 97/18185 and 25 whose structure is represented in Figure 2).

0.36 mmol of 2-methylmercapto-2-imidazolinium iodide is dissolved in 0.36 ml of 1 N sodium hydroxide in a round-bottomed flask equipped with a bubbler and a

magnetic bar. 0.36 mmol of RPR 122766A, previously dissolved in 1.44 ml of 1 N sodium hydroxide, 5 ml of water and 4 ml of ethanol is added to this solution. The mixture is kept stirring until the evolution of 5 methyl mercaptan stops (24 hours). The mixture is then concentrated to dryness in a rotary evaporator. The dry extract is solubilized with a solution of water (4 ml), ethanol (4 ml) and trifluoroacetic acid (0.5 ml). This solution is injected in two portions in preparative 10 HPLC.

10 HPLC.

The fractions of interest (determined by analytical HPLC) are grouped together, frozen and freeze-dried. 213 mg (0.1727 mmol) of salified product are finally obtained.

15 Yield: Y = 48%

HPLC_{analytical}: Rt = 8.90 minutes.

¹H NMR spectrum (400 MHz, (CD₃)₂SO-d₆ with addition of a few drops of CD₃COOD-d₄, δ in ppm) : 0.87 (t, J = 7 Hz, 6H : CH₃ of the fatty chains) ; from 1.15 to 1.40 (mt, 20 44H : central (CH₂)₁₁ of the fatty chains) ; 1.45 and 1.55 (2 mts, 2H each : 1 CH₂ of each fatty chain) ; 1.65 (mt, 4H : the two central CH₂ of the butyl) ; from 1.80 to 1.95 (mt, 4H : central CH₂ of the propyls) ; from 2.80 to 3.05 (mt, 10H : the 2 NCH₂ of the butyl - the 2 25 NCH₂ of one of the 2 propyls - and 1 of the 2 NCH₂ of the other propyl) ; 3.24 (mt, 6H : the other NCH₂ of the other propyl and NCH₂ of the fatty chains) ; 3.56 (s, 2H : NCH₂CON) ; 3.62 (s, 4H : NCH₂CH₂N) ; 4.02 (d, J = 5

Hz, 2H : CONCH₂CON of the glycyl).

MH⁺ = 777

Example 4: Synthesis of compound 4 (2-(3-[bis-[3-(4,5-dihydro-1H-imidazol-2-ylamino)propyl]amino]-5-propylamino)-N-ditetradecylcarbamoylmethylacetamide) by the method for synthesizing building blocks.

a) SYNTHESIS OF BOC-GLY-DITETRADECYLAMINE (1)

Boc-Gly is coupled to ditetradecylamine according to method A described in the "Materials and Methods" section. The yield is 93%.

TLC: R_f = 0.9 (CHCl₃/MeOH, 9:1)

MH⁺: 567

b) SYNTHESIS OF [Z-NH(CH₂)₃]₂-N-(CH₂)₃-NH-Boc-CH₂-COOH (2)

15 1) Synthesis of NC-(CH₂)₂-NH-Boc-CH₂-COOH (3)
The amine of N-(cyanoethyl)glycine is protected with a Boc group according to method D described in the "Materials and Methods" section. The yield is 98%.

20 TLC: R_f = 0.66 (CHCl₃/MeOH, 8:2)

MH⁺: 229

2) Synthesis of NH₂-(CH₂)₃-NH-Boc-CH₂-COOH (4)

The product (3) is hydrogenated according to method F described in the "Materials and Methods" section.

25 TLC: R_f = 0.12 (CHCl₃/MeOH, 6:4)

MH⁺: 233

3) Synthesis of $[\text{NC}(\text{CH}_2)_3]_2\text{-N-}(\text{CH}_2)_3\text{-NH-Boc-CH}_2\text{-}$

GOOF

(5)

The product (4) is dicyanoethylated according to method E described in the "Materials and Methods" section. The yield is 50%.

TLC: $R_f = 0.75$ (CHCl₃/MeOH, 6:4)

MH⁺: 339

4) Synthesis of $[Z\text{-NH-}(\text{CH}_2)_3]_2\text{-N-}(\text{CH}_2)_3\text{-NH-Boc-CH}_2\text{-}$

COOH

(2)

10 The product (5) is hydrogenated according to

method F described in the "Materials and Methods" section.

TLC: $R_f = 0.14$ (CHCl₃/MeOH, 6:4)

Next the amines are protected with Z groups

15 according to method D described in the "Materials and Methods" section. The crude product is purified on a silica column ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 8:2). A white foam is obtained with a yield of 66% relative to the product (4).

20 TLC: $R_f = 0.42$ (CHCl₃/MeOH, 6:4)

MH⁺: 615

c) SYNTHESIS OF $[Z\text{-NH}(\text{CH}_2)_3]_2\text{-N-}(\text{CH}_2)_3\text{-NH-BOC-CH}_2\text{-}$

$$\text{COGlyN}[(\text{CH}_2)_{13}-\text{CH}_3]_2$$

(6)

The Boc of the product (1) is cleaved
25 according to method B described in the "Materials and
Methods" section.

HPLC: $R_t = 12.86$ min, ($H_2O/MeCN : 3$ min [40/60], 3-20 min [0/100], 35 min [0/100]).

The product obtained is coupled with the product (2) according to method A described in the "Materials and Methods" section. The yield is 75% after purification on a silica column (CH₂Cl₂/MeOH, 8:2).

5 TLC: R_f = 0.86 (CHCl₃/MeOH, 8:2)

HPLC: R_t = 17.44 min, (H₂O/MeCN : 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

d) SYNTHESIS OF [NH₂(CH₂)₃]₂-N-(CH₂)₃-NH-Boc-CH₂-COGlyN[(CH₂)₁₃-CH₃]₂

(7)

10 The Z groups are cleaved according to method C described in the "Materials and Methods" section. A yield of 40% is obtained relative to the product (6).

HPLC: R_t = 9.62 min, (H₂O/MeCN : 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

15 MH⁺: 795.

e) SYNTHESIS OF COMPOUND 4

(8)

2-Methylthiocimidazoline hydriodide reacts with the product (7) according to method G described in the "Materials and Methods" section. Boc is cleaved according to method B described in the "Materials and Methods" section. The product is purified by preparative HPLC and the fractions analysed by HPLC. The yield is 34%.

HPLC: R_t = 10.07 min, (H₂O/MeCN : 3 min [40/60], 3-20 min [0/100], 35 min [0/100]).

¹H NMR spectrum (400 MHz, (CD₃)₂SO-d₆ at a temperature of 383 K, d in ppm) : 0.92 (t, J = 7 Hz, 6H : CH₃ of the fatty chains) ; from 1.25 to 1.45 (mt, 44H : central

(CH₂)₁₁ of the fatty chains) ; 1.57 (mt, 4H : 1 CH₂ of each fatty chain) ; from 1.70 to 1.90 (mt, 6H : central CH₂ of the propyls) ; from 2.50 to 3.40 (mt, 16H : the 2 NCH₂ of the propyls and the NCH₂ of the fatty chains) ; 5 3.68 (s, 8H : the 2 NCH₂CH₂N) ; 3.72 (broad s, 2H : NCH₂CON) ; 4.06 (s, 2H : CONCH₂CON of the glycyl).
MH⁺ : 831

B\USE OF THE TRANSFECTION AGENTS ACCORDING TO THE INVENTION

10 Example 5: Preparation of nucleolipid complexes

This example illustrates the preparation of transfer agent/DNA complexes.

15 The cationic lipid used in this example and in the following examples is compound 1 (Figure 1) in solution in chloroform. 10 nmol quantities of compound 1 (that is to say 11.8 µg of compound 1) per µg of DNA were used. In some cases, a neutral co-lipid, cholesterol or DOPE, is previously mixed with the cationic lipid. A fine lipid film forms when the 20 chloroform is evaporated with the aid of a slight stream of argon, and then it is rehydrated in a mixture of 5% dextrose and 20 mM sodium chloride overnight at 4°C. The samples are then treated with ultrasound for 5 minutes, heated at 65°C for 30 minutes and finally 25 treated again with ultrasound for 5 minutes. Lipid suspensions are thus obtained which are stored at 4°C until they are used.

The DNA used is the plasmid pXL2774 (Figure

CODEX 00000000000000000000000000000000

3) in solution in a mixture of 5% dextrose and 20 mM sodium chloride at a concentration of 0.5 mg/ml or 1.0 mg/ml. The plasmid pXL2774 has the following characteristics:

5 - level of endotoxins less than 50 EU/mg,
 - level of supercoiled DNA greater than 60%,
 - content of RNA, that is to say of mRNA, tRNA and
 ribosomal RNA (determined by HPLC) less than 5%,
 - level of chromosomal DNA less than 1%,
10 - protein content less than 1%,
 - osmolarity less than 15 mosmol/kg.

The transfection agent/DNA complexes are prepared by rapidly mixing equal volumes of DNA solution and lipid suspension as described above. The 15 quantity of lipid complexed with the DNA varies from 0.5 nmol/ μ g of DNA to 12 nmol/ μ g of DNA.

Example 6: Behaviour of the complexes formed at different charge ratios

This example illustrates the behaviour of the 20 transfection agent/DNA complexes at different charge ratios. The impact of the addition of a neutral co-lipid is also illustrated.

The size of the complexes was first of all analysed by measuring the hydrodynamic diameter by 25 dynamic light scattering (Dynamic Laser Light Scattering) with the aid of a Coulter N4plus apparatus. The samples are diluted 20-fold in a solution containing 5% dextrose and 20 mM sodium chloride NaCl

in order to avoid multiple diffusions. The effect of the cycloamidine head, of the lipid composition and of the transfer agent/DNA charge ratio on the size of the complexes was thus studied.

5 As a general rule, three possible phases can
be distinguished when the transfer agent/DNA charge
ratio is increased. These three phases determine the
therapeutic potential of the agents for transferring
nucleic acids in question. Figures 4 and 5 illustrate
10 these phases for different cationic lipids: RPR 120535,
RPR 121650 (which have both been described in patent
application WO 97/18185), and compound 1 according to
the invention.

At a low charge ratio, the DNA is not saturated with the cationic lipid. Naked DNA still remains, and the complexes are negatively charged overall. The particles are small in size (between 100 and 300 nm). This phase is called "phase A".

The fact that the DNA is not completely
20 saturated with the cationic lipid means that the DNA is
not completely protected by the lipid. The DNA can
therefore be subjected to degradation by enzymes
(DNases). Moreover, since the complexes are negative
overall, the crossing of the cell membrane is
25 difficult. For these reasons, the nucleolipid complexes
of phase A are relatively inactive.

At an intermediate charge ratio, the DNA is completely saturated with the cationic lipid, and the

complexes are neutral or slightly positive overall. This phase is unstable because the ionic repulsions are minimal and a "crosslinking" phenomenon can occur. The size of the particles is well above the limit of 5 detection by dynamic light scattering (much greater than 3 μm). This unstable phase is called "phase B".

Such a size of complexes is not suited to uses by injection. However, although the RPR 120535/DOPE/DNA complex at a charge ratio of 5 forms large 10 aggregates which precipitate, it has been found to be active in gene transfer. Thus, the complexes are not necessarily inactive in phase B, but they are only in a formulation which is not appropriate for their injection for pharmaceutical purposes.

15 At a relatively high charge ratio, the DNA is oversaturated with the cationic lipid, and the complexes are positive overall. Because of the strong repulsions between the positive charges, this phase is stable. It is designated by the name "phase C".

20 Unlike phase C, the nucleolipid complexes are in a form such that the DNA is very well protected against enzymes, and their overall positive charge facilitates the crossing of the cell membrane of an anionic nature. The phase C complexes are therefore 25 particularly suited to use for the transfer of nucleic acids into cells.

In addition to the cycloamidine head of the cationic lipid, the use of a neutral co-lipid has a

strong impact on the stability of the complexes, as is illustrated in Figure 4 and Figure 5. The co-lipids added in these examples are either DOPE (cationic lipid/DOPE = 3/2), or cholesterol (cationic lipid/cholesterol = 3/1). In general, the addition of the neutral co-lipid increases the instability of the complexes, which causes an increase in the quantity of cationic lipid required to obtain phase C.

It should be noted that the values of the 10 charge ratio which delimit the three phases A, B and C depend on the cationic lipid used. Thus, these values can vary very widely from one lipid to another.

In a second instance, the affinity of the 15 transfer agents for the DNA as a function of the charge ratio was studied. For that, the reduction in fluorescence after the addition of 3 μ g of ethidium bromide (EtBr) was measured. Indeed, the replacement of the ethidium bromide of the DNA by the lipid is an indication of binding to the DNA.

20 The formulations used are diluted 20-fold to a final concentration of 25 μ g of DNA/ml. The relative fluorescence measured for naked DNA is defined as being 100%. The level of binding with the agent for transferring nucleic acids is represented by the 25 reduction in the relative fluorescence of the sample. Figures 4 and 5 show that the fluorescence decreases when the charge ratio increases, which means that a greater quantity of cationic lipid is available to bind

to the DNA (the more the fluorescence decreases, the more a greater quantity of cationic lipid binds to the DNA until saturation is reached).

In this manner, it has thus been shown that
5 the affinity of the cationic lipid for the DNA is
determined by the cycloamidine head, but not by the
addition of a co-lipid.

Example 7: Transfection *in vitro*

This example illustrates the capacity of the transfer agents according to the invention to transfect DNA into cells *in vitro*, compared with naked DNA.

24-well microplates are inoculated with 60,000 HeLa cells (ATCC) per well, and transfected 24 hours later. Complexes containing 1 μ g of DNA are 15 diluted in 0.5 ml of DMEM culture medium (Gibco/BRL) in the absence of serum, and then added to each well. The cells are incubated at 37°C for 4 hours. The medium containing the complexes is then removed and replaced with a mixture of DMEM and 10% foetal calf serum. Next, 20 the cells are again cultured for 24 hours. Finally, the cells are lysed and tested using a luciferase test kit (Promega) and a Dynex MLX luminometer.

The results indicated in Figure 6 underline the difference between the performance of the naked DNA compared with the compound 1/DNA complexes of the invention which are completely saturated: no luciferase activity could be detected (sensitivity of the apparatus less than 1 pg per well) after transfection

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in vitro of naked DNA, whereas the gene transfer activity of the complexes according to the invention varies from 200 pg/well to 8000 pg/well.

This example therefore clearly shows the
5 advantageous use of the compounds according to the
invention for the transfection of cells *in vitro*.

Example 8: Transfection *in vivo*

This example illustrates the capacity of the transfer agents according to the invention to transfet 10 DNA into cells *in vivo*, compared with naked DNA and a known cationic lipid, RPR 120531 described in application WO 97/18185 and whose structure is represented represented in Figure 2.

The gene transfer *in vivo* was performed on
15 Balb/C mice by intramuscular or intravenous
administration. The formulations which were compared
are formulations of naked DNA, formulations containing
RPR 120531, or formulations containing compound 1
according to the invention.

20 In the case of intramuscular injections, each
mouse received 30 μ l of formulation containing 15 μ g of
DNA in the anterior muscle of the tibia. The tissues
are recovered 7 days after the injection, they are
frozen and stored at -80°C while waiting to perform the
25 luciferase activity tests. The measurements of
luciferase activity are carried out as in Example 6.

In the case of injections by the intravenous route, each mouse received 200 μ l of formulation

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containing 50 μ g of DNA. The tissues are recovered in this case 24 hours after the injection and then frozen and stored in the same manner as above.

The results of gene transfer *in vivo* are presented [lacuna] Figure 7 and Figure 8. The ratio between compound 1 and the DNA is 10 nmol/ μ g of DNA. The ratio between RPR 120531 and the DNA is 4 nmol/ μ g of DNA.

Figure 7 illustrates the *in vivo* activity of compound 1 according to the invention compared with naked DNA and with RPR 120531. It is observed that the levels of luciferase activity are equivalent between naked DNA and compound 1, the latter having, in addition, a highly improved activity compared with RPR 120531. The transfer mechanisms involved appear to be different between naked DNA and the use of compound 1 according to the present invention. Indeed, the complexes according to the invention used do not contain free DNA (phase C) and furthermore, their results *in vitro* are considerably greater than those for naked DNA.

Figure 8 compares the activity of compound 1 according to the invention, of naked DNA and of RPR 120531, by the intravenous route and by the intramuscular route.

It is observed that the transfection efficiency is roughly equivalent by the intravenous route for the two cationic lipids. On the other hand,

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by the intramuscular route, the transfection efficiency of compound 1 according to the invention is quite considerably greater.

Compared with naked DNA, compound 1 exhibits 5 transfection by the intravenous route, in addition to transfection by the intramuscular route which is at least equivalent.

It therefore appears that the nucleic acid transfer efficiency *in vivo* of compound 1 according to 10 the invention is greater than that of naked DNA and of RPR 120531 overall.

Finally, it appears that the complexes according to the invention have the advantage, compared 15 with transfection of naked DNA, of protecting the DNA from degradation by nucleases, thus contributing to a significant improvement in the stability of the formulations. The compounds of the present invention can also be used to protect DNA from damage during 20 freeze-drying, improving here again the stability of the formulations.

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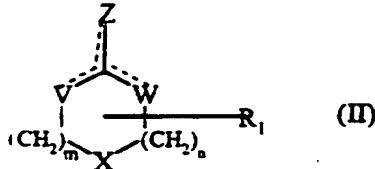
CLAIMS

1. Agent for transferring nucleic acids, in D, L or DL form, as well as its salts, of general formula (I):

5 CA-Rep-R (I)

for which:

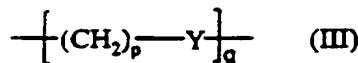
1. CA represents a cycloamidine head and its mesomeric forms of general formula (II):



for which:

10 • m and n are integers, independent of each other, between 0 and 3 inclusive and such that $m+n$ is greater than or equal to 1,

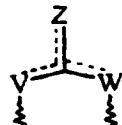
 • R_1 represents a group of general formula (III):



for which p and q are integers, independent of each other, between 0 and 10 inclusive, and Y represents a carbonyl, amine, methylamine or methylene group, it being possible for Y to have different meanings within the different $[(\text{CH}_2)_p\text{-Y}]$ groups, and it being understood that R_1 may be linked to any atom of the general formula

(III),

- X represents a group NR_2 or CHR_2 , R_2 being either a hydrogen atom or the group R_1 as defined above,

- the group  represents:

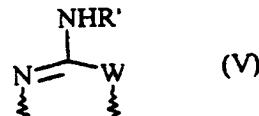
5 * 1st case: a group of general formula (IV):



for which W represents CHR''' or NR''' , and R'' and R''' represent, independently of each other, a hydrogen atom, a methyl, or the group R_1 as defined above, it being understood that R'' and R''' cannot be R_1 at the

10 same time, or

* 2nd case: a group of general formula (V):



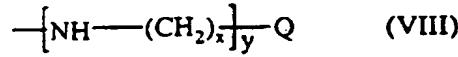
for which W represents CHR''' or NR''' , and R' and R''' represent, independently of each other, a hydrogen atom, a methyl or the group R_1 as defined above, it being understood that R' and R''' cannot be R_1 at the 15 same time,

-----independent of each other and may have different meanings,

• R₄ is defined in the same way as R₃ or represents a group CA as defined above, it being understood that the 5 CA groups are independent of each other and may be different, and

3. R is linked to the carbonyl functional group of the group Rep of general formula (VI), or if Rep is absent, R is linked directly to CA, and 10 represents:

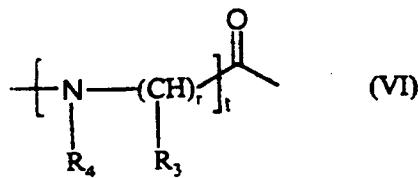
* either a group of formula NR₆R, for which R₆ and R, represent, independently of each other, a hydrogen atom or an optionally fluorinated, linear or branched, saturated or unsaturated aliphatic radical containing 1 15 to 22 carbon atoms, with at least one of the two substituents R₆ or R, different from hydrogen and the other containing between 10 and 22 carbon atoms,
 * or a steroid derivative,
 * or a group of general formula (VIII):



20 for which x is an integer between 1 and 8 inclusive, y is an integer between 1 and 10 inclusive, and either Q represents a group C(O)NR₆R₇, for which R₆ and R₇ are as defined above, or Q represents a group C(O)R₈ for which R₈ represents a group of formula (IX):

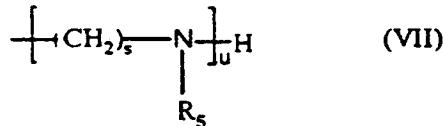
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2. Rep is absent or is a spacer of general formula (VI):

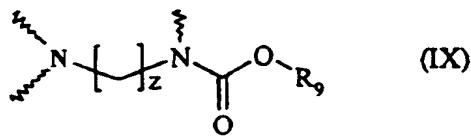


whose nitrogen atom is attached to CA, and:

- t is an integer between 0 and 8 inclusive,
- 5 • r is an integer between 0 and 10 inclusive, it being possible for r to have different meanings within the different $-\text{NR}_4-(\text{CH})_r-$ groups,
- R_3 , which may have different meanings within the different $\text{NR}_4-(\text{CH})_r\text{R}_3$ groups, represents a hydrogen atom, a methyl group or a group of general formula
- 10 (VII):



for which u is an integer between 1 and 10 inclusive, s is an integer between 2 and 8 inclusive which may have different meanings within the different $-(\text{CH}_2)_s-\text{NR}_5$ groups, and R_5 is a hydrogen atom, a group CA as defined above, it being understood that the CA groups are independent of each other and may be different, or a group of general formula (VII), it being understood that the groups of general formula (VII) are



for which z is an integer between 2 and 8 inclusive, and R_9 is an optionally fluorinated, saturated or unsaturated aliphatic radical containing 8 to 22 carbon atoms, or a steroid derivative,

5 or R_8 represents a group $-O-R_9$, for which R_9 is as defined above.

2. Agent for transferring nucleic acids according to claim 1, characterized in that, in the group of formula (VI), R_3 and R_4 represent hydrogen atoms or R_4 represents a hydrogen atom and R_3 is a group of formula (VII) as defined in claim 1 for which R_5 represents a cycloamidine head CA.

15 3. Agent for transferring nucleic acids according to claim 1, characterized in that the cycloamidine head CA of formula (II) comprises 5, 6, 7 or 8 members.

4. Agent for transferring nucleic acids according to claim 1, characterized in that, in formula (III), p and q are chosen, independently of each other, 20 from 2, 3 or 4.

5. Agent for transferring nucleic acids according to claim 1, characterized in that the groups R_6 and R_7 are identical or different and each represent optionally fluorinated, linear or branched, saturated

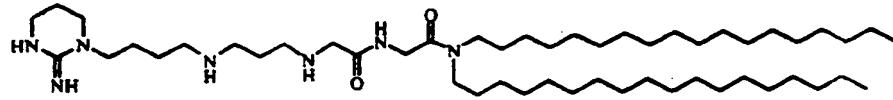
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or unsaturated aliphatic chains containing 10 to 22 carbon atoms.

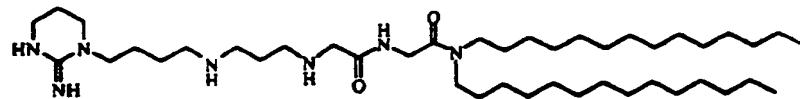
6. Agent for transferring nucleic acids according to claim 1, characterized in that the groups 5 R_6 and R_7 are identical or different and each represent optionally fluorinated, linear or branched, saturated or unsaturated aliphatic chains containing 12, 14, 16, 17, 18 or 19 carbon atoms.

7. Agent for transferring nucleic acids 10 according to claims 1 and 5, characterized in that the steroid derivative is chosen from cholesterol, cholestanol, 3- α -5-cyclo-5- α -cholestane-6- β -ol, cholic acid, cholesteryl formate, cholestanyl formate, 3 α ,5-cyclo-5 α -cholestane-6 β -yl formate, cholesterylamine, 15 6-(1,5-dimethylhexyl)-3 α ,5 α -dimethylhexadecahydrocyclopenta[a]cyclopropane[2,3]cyclo-penta[1,2-f]naphthalen-10-ylamine, or cholestanylamine.

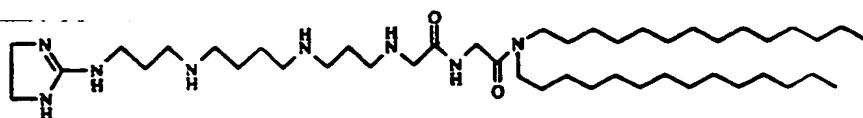
8. Agents for transferring nucleic acids according to claim 1 of formulae:



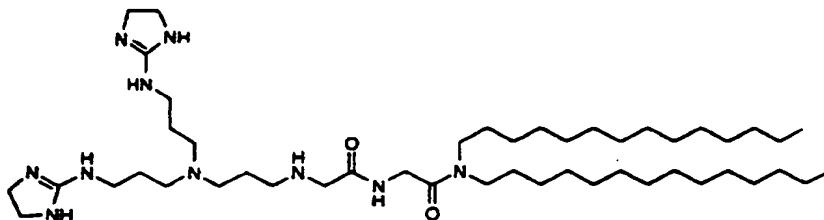
1



2



3



4

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9. Method of preparing agents for transferring nucleic acids according to claims 1 to 8, characterized in that the synthesis of building blocks carrying the cycloamidine functional group(s) is 5 carried out and then these building blocks are grafted onto lipids equipped with spacers.

10. Method of preparing agents for transferring nucleic acids according to claims 1 to 8, characterized in that the synthesis of the 10 lipopolyamines is carried out and then the cyclization into a cycloamidine head is carried out.

11. Composition characterized in that it comprises an agent for transferring nucleic acids according to one of claims 1 to 8 and a nucleic acid.

15 12. Composition according to claim 11, characterized in that it comprises, in addition, one or more adjuvants.

13. Composition according to claim 12,

characterized in that the adjuvant is one or more neutral lipids.

14. Composition according to claims 12 and 13, characterized in that the neutral lipids are lipids 5 containing two fatty chains.

15. Composition according to claims 12 to 14, characterized in that the neutral lipids are natural or synthetic lipids which are zwitterionic or lacking ionic charge under physiological conditions, 10 chosen for example from dioleoylphosphatidyl-ethanolamine (DOPE), oleoylpalmitoyl-phosphatidylethanolamine (POPE), di-stearoyl, -palmitoyl, -myristoylphosphatidylethanolamines as well as their derivatives which are N-methylated 1 to 3 15 times, phosphatidylglycerols, diacylglycerols, glycosyldiacylglycerols, cerebrosides (such as in particular galactocerebrosides), sphingolipids (such as in particular sphingomyelins) or asialogangliosides (such as in particular asialoGM1 and GM2).

20 16. Composition according to claim 12, characterized in that the adjuvant is a compound involved directly or otherwise in the condensation of the nucleic acid.

25 17. Composition according to claim 16, characterized in that the adjuvant is derived, as a whole or in part, from a protamine, a histone or a nucleolin and/or from one of their derivatives, or alternatively consists, as a whole or in part, of

peptide units (KTPKKAKKP) and/or (ATPAKKAA), it being possible for the number of units to vary between 2 and 10 and to be repeated continuously or otherwise.

18. Composition according to claims 11 to 5 17, characterized in that it contains, in addition, one or more nonionic surfactants in a sufficient quantity to stabilize the size of the particles of nucleolipid complexes.

19. Composition according to claims 11 to 10 18, characterized in that it comprises a pharmaceutically acceptable vehicle for an injectable formulation.

20. Composition according to claims 11 to 18, characterized in that it comprises a 15 pharmaceutically acceptable vehicle for application to the skin and/or the mucous membranes.

21. Use of a transfer agent according to one of claims 1 to 8 for transferring nucleic acids into cells.

20 22. Use of an agent for transferring nucleic acids according to one of claims 1 to 8 for transferring nucleic acids into cells by the intramuscular route.

23. Method of transferring nucleic acids 25 into cells, characterized in that it comprises the following steps:

(1) bringing the nucleic acid into contact with a transfer agent as defined in claims 1 to 8 to

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form a complex, and

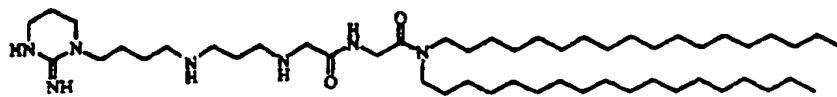
(2) bringing the cells into contact with the complex formed in (1).

24. Method of transferring nucleic acids

5 into cells according to claim 23, characterized in that
the said nucleic acid and/or the said transfer agent
are previously mixed with one or more adjuvants.

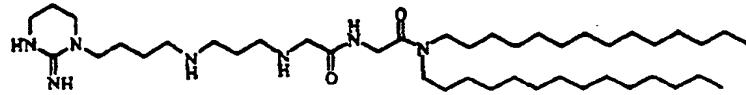
6 10 10 13 15 13 14 15 " 10 15 11 12 9 13

FIG. 1/8



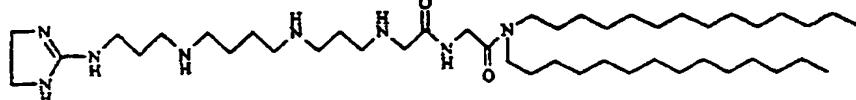
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N-dioctadecylcarbamoylmethyl-2-{3-[4-(2-iminotetrahydropyrimidin-1-yl)butylamino]propylamino}acetamide



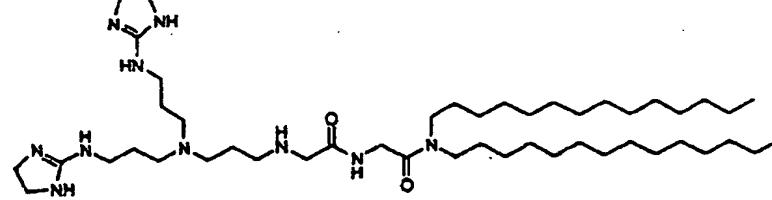
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N-ditetradecylcarbamoylmethyl-2-{3-[4-(2-iminotetrahydropyrimidin-1-yl)butylamino]propylamino}acetamide



3

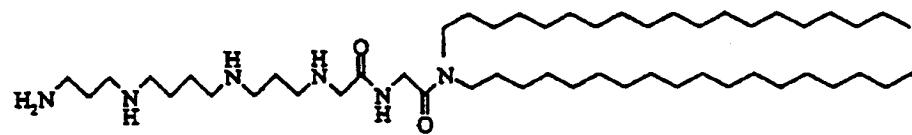
2-(3-{4-[3-(4,5-dihydro-1H-imidazol-2-ylamino)propylamino]butylamino}-N-ditetradecylcarbamoylmethylacetamide



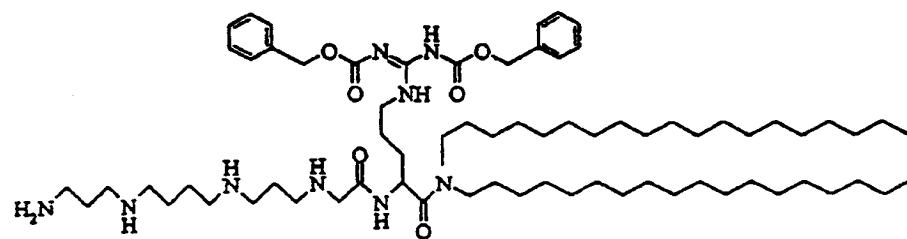
4

2-(3-{bis-[3-(4,5-dihydro-1H-imidazol-2-ylamino)propyl]amino}propylamino)-N-ditetradecylcarbamoylmethylacetamide.

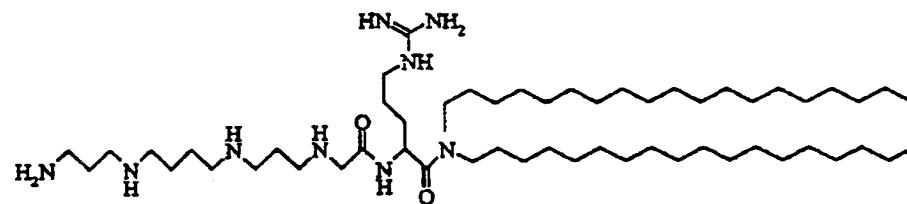
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FIG. 2/8

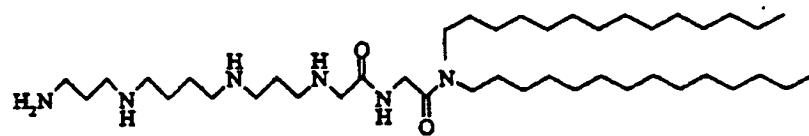
RPR 120535



RPR 121650



RPR 120531



RPR 122766

60085645 1990

FIG. 3/8

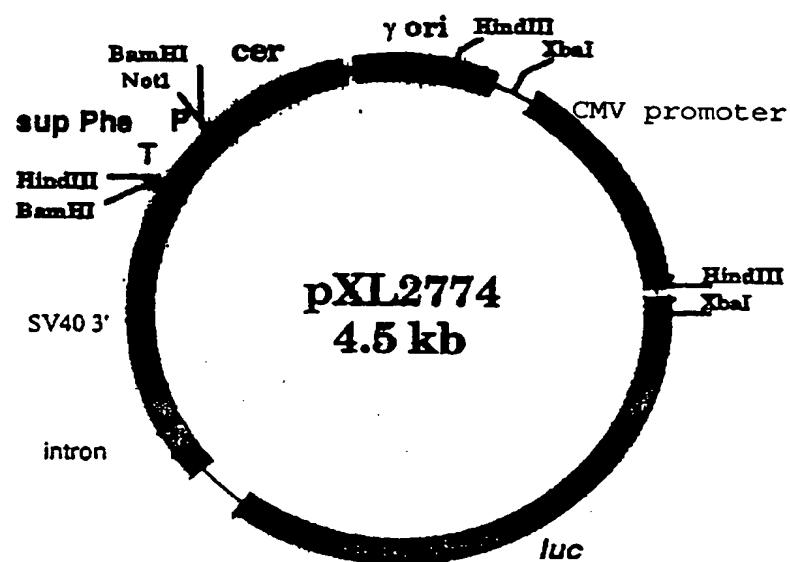


FIG. 4/8

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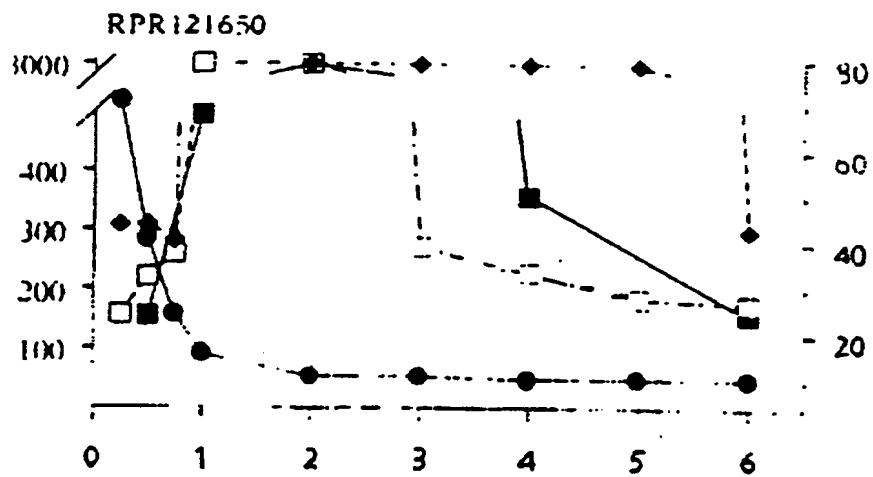
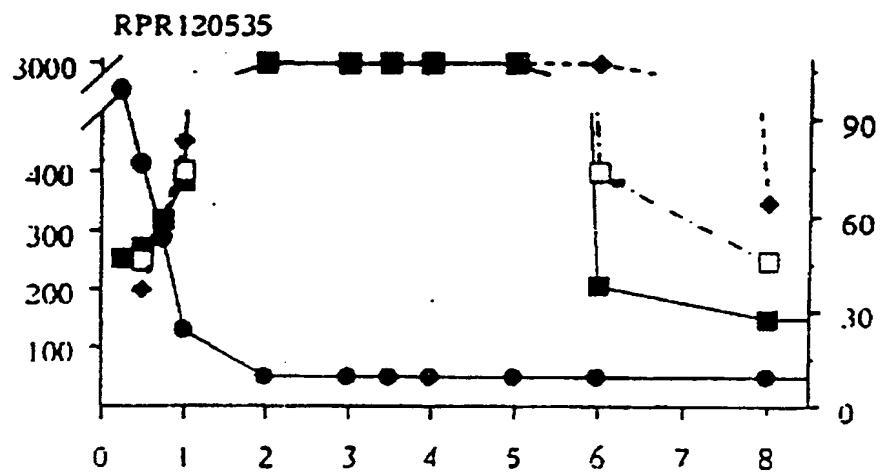
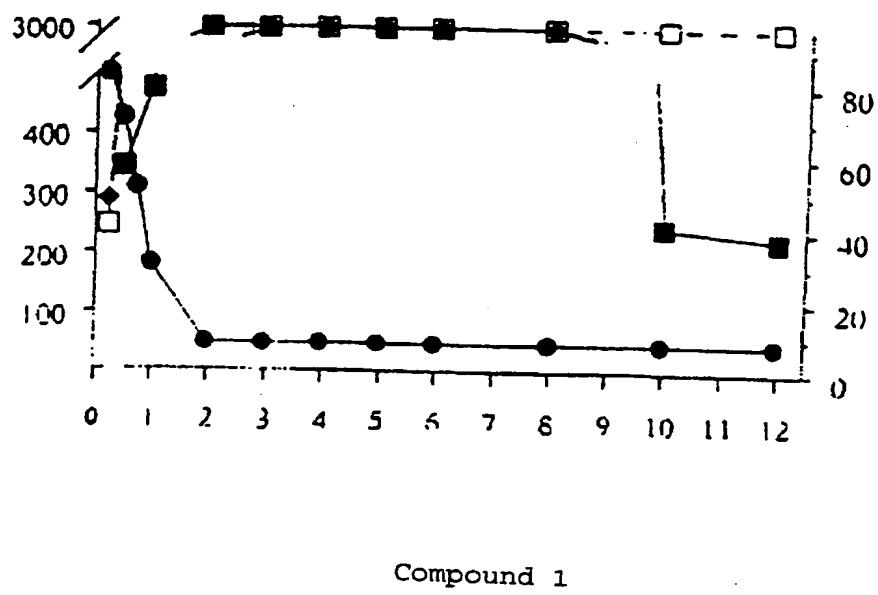
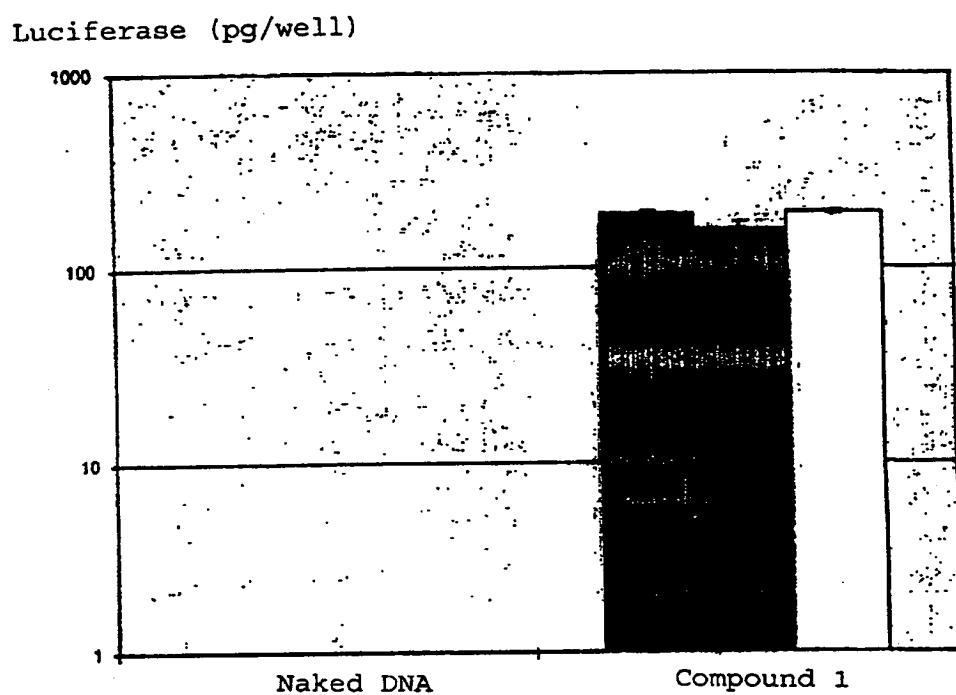


FIG. 5/8

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FIG. 6/8



1600 1615 1630 1645 1655

8163150 " 5-18558005

7

FIG. 7/8

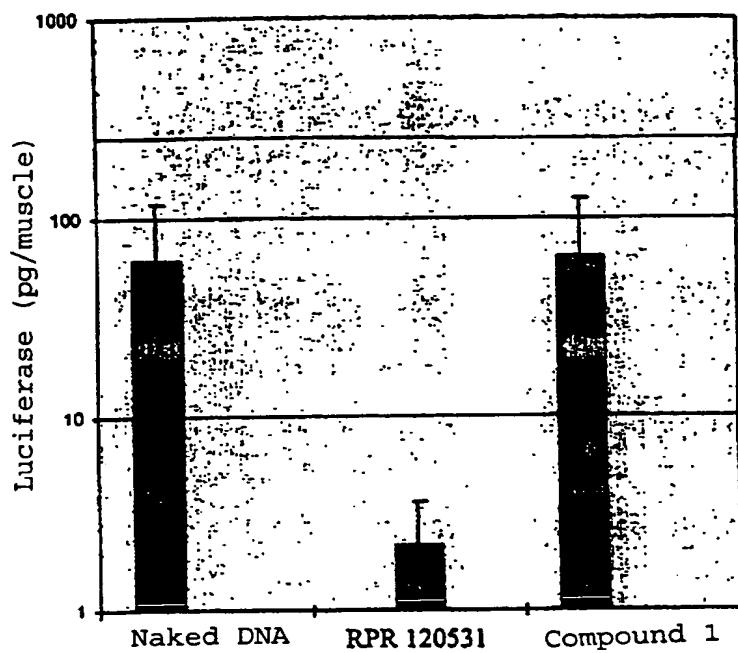


FIG. 8/8

